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DOI: https://doi.org/10.1007/s12035-018-1314-3

Originally published at:
Jawaid, Ali; Woldemichael, Bisrat T; Kremer, Eloïse A; Laferriere, Florent; Gaur, Niharika; Afroz, Tariq; Polymenidou, Magdalini; Mansuy, Isabelle M (2019). Memory decline and its reversal in aging and neurodegeneration involve miR-183/96/182 biogenesis. Molecular Neurobiology, 56(5):3451-3462.
DOI: https://doi.org/10.1007/s12035-018-1314-3

Molecular Neurobiology
https://doi.org/10.1007/s12035-018-1314-3

Memory Decline and Its Reversal in Aging and Neurodegeneration Involve miR-183/96/182 Biogenesis

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Received: 11 May 2018 / Accepted: 9 August 2018

Abstract

Aging is characterized by progressive memory decline that can lead to dementia when associated with neurodegeneration. Here, we show in mice that aging-related memory decline involves defective biogenesis of microRNAs (miRNAs), in particular miR-183/96/182 cluster, resulting from increased protein phosphatase 1 (PP1) and altered receptor SMAD (R-SMAD) signaling. Correction of the defect by miR-183/96/182 overexpression in hippocampus or by environmental enrichment that normalizes PP1 activity restores memory in aged animals. Regulation of miR-183/96/182 biogenesis is shown to involve the neurodegeneration-related RNA-binding proteins TDP-43 and FUS. Similar alterations in miR-183/96/182, PP1, and R-SMADs are observed in the brains of patients with amyotrophic lateral sclerosis (ALS) or frontotemporal lobar degeneration (FTLD), two neurodegenerative diseases with pathological aggregation of TDP-43. Overall, these results identify new mechanistic links between miR-183/96/182, PP1, TDP-43, and FUS in age-related memory deficits and their reversal.

Keywords Memory · microRNA · Protein phosphatase 1 · Dementia · TDP-43 · FUS

Introduction

Aging is often associated with a progressive deterioration of learning abilities, attention and memory formation [3]. These deficits vary in their manifestation and severity, ranging from occasional lapses in memory to widespread deficits in multiple cognitive domains. Longitudinal studies of brain functioning in the elderly have revealed progressive deficits especially in episodic long-term memory [14]. Further, marked memory impairment together with other neuropsychiatric symptoms, collectively called dementia, is a cardinal feature of aging-associated neurodegenerative disorders. Animal models of such disorders have deficits in memory prior to any obvious indication of neuronal death, implicating mechanisms independent of neurodegeneration [11, 39]. Consistently, an epigenetic mechanism of memory deficit was recently reported in a mouse model of Alzheimer disease (AD, [13]).

Alterations in multiple signaling pathways, particularly pathways related to neuronal plasticity and synaptic functions have been implicated in age-related cognitive decline. MicroRNAs (miRNAs) are potential important players in the mechanisms underlying memory loss associated with aging and neurodegeneration [23]. Comparison of deep sequencing data from whole brain in young and aged mice have shown differential expression of 93 miRNAs with aging [18]. Similarly, proteins pathologically aggregated in neurodegenerative disorders, such as TAR DNA-binding protein of 43 kDa (TDP-43) and fused in sarcoma (FUS), regulate the activity of miRNAs biogenesis machinery, in particular Drosha and Dicer [9, 20]. Impaired miRNA biogenesis is therefore an important candidate mechanism for cognitive dysfunction in neurodegenerative disorders with TDP-43 and FUS pathologies.

We previously showed that the nuclear biogenesis of the miRNAs cluster miR-183/96/182, a cluster upregulated in the

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12035-018-1314-3) contains supplementary material, which is available to authorized users.

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Published online: 20 August 2018

adult brain by learning, is regulated by the memory suppressor protein phosphatase 1 (PP1) in a transcription-independent manner [42].As pathological protein aggregates in neurodegenerative disorders are often phosphorylated, for instance, TDP-43 aggregates in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD, [31]), FUS aggregates in FTLD [32], and tau aggregates in FTLD and other tauopathies [40], an alteration of phosphatases has been proposed to be implicated in these disorders. Further, TDP-43 and FUS also regulate the expression or activity of some phosphatases [33, 38]). Thus, a role for PP1 in the regulation of memory-related miRNAs and an implication of phosphatases in neurodegenerative disorders suggest that abnormal PP1 activity leading to impaired biogenesis of memory-related miRNAs could contribute to memory impairment in neurodegenerative conditions.

Here, we demonstrate that in the aged brain, increased nuclear PP1 impairs the biogenesis of miR-183/96/182 that correlates with memory decline. Correction of this impairment by reduction in PP1 activity or miR-183/96/182 increase in the hippocampus restore memory functions. We also show that PP1 is increased and miR-183/96/182 decreased in the brain of ALS and FTLD patients postmortem. Finally, we show that TDP-43 and FUS regulate the biogenesis of miR-183/96/182 (in a PP1-dependent manner for TDP-43), and involve receptor SMAD proteins (R-SMADs) as likely mediators of the interaction between miR-183/96/182 and PP1.

Materials and Methods

Animals

Young (3 months old) and aged (18 months old) C57Bl/6 mice were purchased from Janvier (France). The mice were group-housed (four per cage) under a reverse 12-h light/dark cycle (25 °C, 55% humidity) with food and water *ad libitum*. Behavioral experiments were conducted during the dark cycle. Experiments and animal maintenance were conducted in compliance with the Federation of Swiss Cantonal Veterinary Office and approved by Zürich Cantonal Veterinary Office (54/2012).

Novel Object Recognition (NOR) Task

Object recognition training was conducted in a rectangular arena (60 cm × 50 cm × 45 cm), with gray, opaque walls, and a translucent plexiglass bottom with an infrared light source placed below. The box was located in a dedicated behavioral room illuminated by a dim light. Before NOR training, each animal was handled daily for 4 min on 4 consecutive days. This was followed by an open field test, which involved placing each mouse in the empty arena for 10 min and measuring overall locomotor activity. An additional habituation to the empty arena was conducted one day before the NOR training. For training (acquisition), three different unfamiliar objects were placed in the center of the arena in a triangular arrangement. Each animal was allowed to explore the objects for a 10-min session. Object memory was tested in 5-min sessions for which one of the familiar objects was replaced with a novel object at 24 h (test 1) and 48 h (test 2) after training. The time each animal spent exploring each object during testing was measured with a video tracking system (View Point Behavior Technology) by an experimenter blinded to group assignment. Object memory was expressed as the proportion of time spent exploring the novel object compared to the time spent exploring all objects (discrimination index). The discrimination index was normalized taking the average value from control animals as 100%. The animal movements were tracked by an infrared camera connected to a tracking software (View Point Behavior Technology). For miRNA expression experiments, NOR controls (identified as "habituation") were subjected to identical procedures without an exposure to objects.

Environmental Enrichment (EE)

Young adult (4–5 months) and aged (19–21 months) C57Bl/6J male mice were either housed in standard cages or in environmentally enriched cages for 4 weeks. Standard housing consisted of a clear polycarbonate standard Aero cages (391 × 199 × 160 mm) containing wood chip bedding and nesting material. Enriched housing consisted of a clear polycarbonate type 2000P cage (610 × 435 × 215 mm) containing wood chip bedding, nesting material, two running wheels, a climbing ladder, a jungle gym, a plumbing pipe, multiple plastic balls, a rubber ball, and a wooden stick.

Cell Culture

Mouse neuroblastoma (N2A) cells were obtained from American Type Cell Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, high glucose), supplemented with 10% (*v*/*v*) FBS (Gibco®) and 1% Antibiotic-Antimycotic (Gibco®). These cells were chosen for their fast growth, easy maintenance and transfection, and potential for neuronal differentiation making them a convenient model for studying miRNA biogenesis. The cells were proven free of mycoplasma contamination through regular tests. Before the start of experiments, the cells were passaged 1:4 and split every 3 days for at least five passages. On transfection day, 150,000–300,000 cells were plated in 6- or 12-well plates. Transfection of a pool of siRNAs targeting TDP-43/FUS/α-synuclein (Flexitube siRNA, Qiagen) or negative control siRNA (All Star negative control, Qiagen) was carried out with HiPerfect® transfection reagent (Qiagen).

Overexpression or inhibition of nuclear PP1 was achieved by expressing plasmids containing PP1γ or NIPP1 open read frame (Origene). Then the cells were returned to the incubator after transfection and grown for 48 h prior to harvest or further treatment. The cells were harvested by removing the medium, washing with ice-cold PBS three times, and lysing with Tri-reagent® (for RNA extraction) or RIPA buffer (for protein extraction) or 0.05% Trypsin-EDTA (for sub-cellular fractionation). All experiments were conducted on at least three replicates from different passage number, and repeated at least three times.

Drug Treatments

Cellular senescence was induced by treating N2a cells with 8 or 16 mM hydroxyurea (HU, Sigma-Aldrich, St. Louis, MO, USA) for an incubation time (37 °C, 5% CO_2) of 12 h. HU treatment was done 12 h prior to cell lysis. H_2O was used as a control. Induction of cellular senescence was confirmed by checking the expression of senescence markers p21 and p53. Primer sequences: p21 (Fwd: TACTTCCTCTGCCCTGCTGC, Rev: GCTGGTCTGCCTCCGTTTT), p53 (Fwd: CACGTACTCTCCTCCCCTCAAT, Rev: AACTGCACAGGGCACGTCTT). Protein phosphatases were inhibited by treating N2a cells with okadaic acid (cell signaling) at two different doses: 20 nM (inhibition of PP1/PP2A) and 0.1 nM (inhibition of PP2A only) [19].

Nuclear PP1 Activity Assays

Nuclear PP1 activity assays were performed as previously described [22]. Briefly, hippocampi were dissected and homogenized in 500 ul of cytoplasmic extraction buffer (Subcellular Protein Fractionation Kit for Tissue, Thermo Fischer scientific), transferred to Pierce Tissue Strainer and centrifuged at 500 g for 5 min. The supernatant containing the cytoplasmic protein fraction was isolated, and the pellet containing the nuclear fraction was incubated on ice for 10 min, and centrifuged at 3000 g for 5 min in 325 ul membrane extraction buffer. The pellet containing the nuclei was resuspended in 110 ul of nuclear extraction buffer (NEB), incubated for 30 min, and centrifuged at 5000*g* for 5 min. The supernatant containing soluble nuclear extracts was desalted using PiBind resin (Innova Biosciences). Phosphatase activity was determined by incubating 20 ul of the isolated nuclear fraction with 0.75 mM RII phosphopeptide substrate (BIOMOL), with or without 5 nM tautomycin (selective inhibitor of PP1 at 5 nM, ENZO Life Sciences) at 30 °C for 10 min. The amount of free phosphate released was measured with BIOMOL green reagent (BIOMOL) at 620 nm and background subtracted. Nuclear PP1 activity was calculated by subtracting the nuclear phosphatase activity with tautomycin from the nuclear phosphatase activity without tautomycin.

Luciferase-Based Pri-miRNA Processing

Pri-miRNA processing assays were conducted as previously described [4, 5]. Briefly, fragments of pri-mir-182 and pri-mir-183 containing the hairpin and 100 bp flanking sequence were amplified from genomic DNA. The PCR products were digested with the respective restriction enzymes and inserted at MCS in pmirGLO vector (Dual Reporter Luciferase Assay System, Promega) downstream to firefly luciferase reporter. Cropping of the hairpin stem-loop of the inserts results in destabilization of the firefly reporter leading to a decrease in firefly luminescence. The unperturbed renilla reporter produces stable luminescence, which serves as internal normalization control. Dual-luciferase reporters with pri-mir-182 and pri-mir-183 were transfected in N2a cells using cationic liposomes (Lipofectamine 2000 reagent, Invitrogen). The cells were lysed 48 h post-transfection with passive lysis buffer (Promega) treatment at room temperature for 10 min. The lysates were then transferred to a 96-well plate, and luciferase activities of firefly and renilla were read through luminometer GloMax 96 (Promega) equipped with dual injections dispersing LAR II (for firefly luciferase quantification) and Glomax (for renilla luciferase quantification) reagents sequentially.

Plasmid Production for miRNA Overexpression

The scAAV2-EF1a-pri-miR-183/96/182-GFP construct was produced by cutting scAAV2-MCS (Cell Biolabs) by BalI/NotI (New England Biolabs). The transgene cassette containing Ef1a promoter (sequence from pEGP-mmu-miR-182 plasmid; Cell Biolabs), engineered truncated (T)-pri-miR-183/96/182, EGFP (from pEGP-mmu-miR-182 plasmid; Cell Biolabs), WPRE motif and 5′-BalI and 3′-NotI adapters was chemically synthesized by GENEWIZ (South Plainfield, USA) and cloned into the scAAV backbone. Sequence of T-pri-miR-183/96/182 is as follows :5′ cctctgcagggtctgcaggctggagagtgtgactcctgtcctgtgtatggcactggtagaattcactgtgaacagtctcagtcagtgaattaccgaagggccataaacagagcagagacagatccgcgagcaccttggagctcctcaccccttctgcctagacctctgtttccaggggtgccagggtacaaagacctcctctgctccttccccagagggcctgttccagtaccatctgcttggccgattttggcactagcacatttttgcttgtgtctctccgctgtgagcaatcatgtgtagtgccaatatgggaaaagcgggctgctgcggccacgttcacctccccccggcatcccataataaaaacaagtatgctggaggcctcccaccatttttggcaatggtagaactcacaccggtaaggtaatgggacccggtggttctagacttgccaactatggtgtaagtgctgagct. The scAAV2-EF1a-pri-miR-183/96/182-GFP allowed the generation of mature miR-183-5p, miR-96-5p, and miR-182-5p sequences annotated in miRBASE v.20 (www.mirbase.org), whose expression was verified both by RT-qPCR and Northern blot analysis in HEK293 cells transfected with scAAV2-EF1a-pri-miR-183/96/182-GFP

plasmid (data not shown). We also verified that expression of these miRNAs in HEK293 cells leads to specific repression of reporter mRNAs bearing miR-183/96/182 sites (data not shown). Control scAAV2-EF1a-control-GFP construct contained fragment of beta-globin intron (sequence from pEGP-mmu-miR-182 plasmid; Cell Biolabs) of length corresponding to T-pri-miR-183/96/182.

scAAV Production

Self-complementary AAV production was performed by triple transfection of HEK 293T cells using polyethylenimine with a plasmid bearing the target sequences between the internal terminal repeats of scAAV2, the AAV-helper plasmid encoding Rep2 and Cap for serotype 8, and the pHGTI-Adeno1 plasmid harboring helper adenoviral genes (both kindly provided by C Cepko, Harvard Medical School, Boston, MA, USA). Vectors were purified using a discontinuous iodixanol gradient (Sigma, Optiprep). Encapsidated DNA was quantified by TaqMan RT-PCR following denaturation of the AAV particles by Proteinase K, and titers were calculated as genome copies (GC) per milliliter.

Stereotaxic Surgery and Intra-Hippocampal Injections

To overexpress miRNAs, virus vectors were prepared as described above. The oligonucleotides were stereotaxically injected into the CA1 region of the hippocampus at a concentration of 1μg/ul, in TurboFect in vivo transfection reagent (Dharmacon). Animals were anesthetized with 3% isoflurane (Attane) and placed in a stereotaxic frame. Anesthesia was maintained with 1.5% isoflurane in 100% oxygen throughout surgery. Injection was carried out by lowering a glass pipette (Blaubrand, cut to a 20-μm inner diameter) filled with virus/oligonucleotides and attached to an injection pump (Stoelting) through a predrilled hole at the following coordinate targeting CA1 region of the hippocampus (from Bregma): AP − 2.0 mm, ML ± 1.5 mm, DV − 1.6 mm. A total of 1 μl (10^9GC/ml) of virus vector or 1.5 μl of TSB oligos was injected into each hippocampus at a rate of 0.2 μl/min. The glass pipette was left in place for an additional 5 min, before it was carefully withdrawn and the wound was closed. The animals were allowed to recover for 14 days in their home cage before behavioral testing.

RNA Extraction and RT-qPCR

Mouse hippocampal tissue was homogenized using TissueLyser (Qiagen) in Trizol reagent (Invitrogen). Total RNA was extracted by phenol-chloroform precipitation. For extraction from cells, the medium was removed, the cells were washed three times with ice cold-PBS, lysed and homogenized by adding Trizol directly to the plates. Subcellular fractionation of nuclear and cytoplasmic RNA was performed using Norgen's Cytoplasmic and nuclear RNA purification kit (Norge BioTek, Canada). One microgram of total RNA was treated with RNase-free DNase (Promega) and reverse transcribed using miScript II RT kit (Qiagen). Miscript primer assays (Qiagen) for mature and precursor miRNAs were used to amplify the respective transcripts from a cDNA pool. For mRNA quantification, Quantitect (Qiagen) gene-specific primers were used. Real-time PCR was performed on LightCycler 480® (Roche). Small nuclear RNA (RNU6) or GAPDH was used as endogenous control and quantification was performed as previously described [37].

Protein Extraction and Western Blotting

Total proteins were extracted from N2a cells using radio immunoprecipitation buffer (RIPA) with 1:1000 protease inhibitor cocktail (Sigma-Aldrich) and 1:500 phenyl methyl sulfonyl fluoride (PMSF). Cells were lysed directly on the culture plate with 100–150 □l RIPA and scraped off with a cell scraper. The lysate was transferred to a micro centrifuge tube and sonicated for five cycles, each comprising 30- s of sonication with 30-s intervals. The resulting mixture was centrifuged for 15 min at 14,000*g* to separate the protein mixture (supernatant) from cellular debris (pellet). Nuclear proteins were extracted from N2a cells as described in the section "PP1 activity assays." Twenty- to forty-microgram proteins were resolved on SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-rad). Membranes were blocked in 3% BSA for 1 hour, and then incubated in primary (overnight at 4 °C) and secondary (1 hour at room temperature) antibodies. They were scanned using Odessey IR scanner (Li-Cor Bioscience), and band intensity was determined and quantified using image analysis software (ImageJ). The following antibodies were used: primary anti-phospho SMAD 1/5 Ser 463/465 (Cell Signaling Technology, mAb 9516), Drosha (Abcam, ab135956), DGCR8 (Abcam, ab35865), Cyclophilin A (Abcam, ab58144), Histone H3 (Abcam, ab 1791), GAPDH (Abcam, ab 9485); secondary—anti-mouse IRDye® goat anti-mouse (LI-COR, 925-32210), and IRDye® goat anti-rabbit (LI-COR, 925-32211).

Human Tissue

Fresh frontal cortices from controls (n = 6-8), sALS (n = 10), and FTLD (n=9-12) brains were fixed with phosphate-buffered 3.65% formaldehyde, embedded in paraffin, cut into 6-μm serial sections and mounted on glass slides. Sections were deparaffinated and rehydrated using a gradient of alcohol. Antigen retrieval was done by boiling the sections in 10-mM citrate buffer (pH 6.0) in a microwave oven. Immunohistochemistry was performed using the avidin-biotin complex (ABC) detection system (Vector Laboratories) and 3,3-diaminobenzidine. Endogenous peroxidases were first quenched with 5% H_2O_2 in methanol for 30 min, and sections

were blocked in 0.1 mol/L Tris with 2% fetal bovine serum for 5 min. Primary antibodies were incubated for 2 h at room temperature. The following primary antibodies were used: affinity-purified rabbit polyclonal anti-TDP-43 (pan TDP-43 pAb 1:4000; ProteinTech), and phosphorylation specific rabbit polyclonal pAb anti-pTDP (pS403/404 1:2000 Cosmobio). After washing, sections were sequentially incubated with biotinylated secondary antibodies for 1 h and avidin-biotin complex for 1 h. Bound antibody complexes were visualized by incubating sections in a solution containing 100 mM Tris, pH 7.6, 0.1% Triton X-100, 1.4 mM diaminobenzidine, 10 mM imidazole, and 8.8 mM H_2O_2. Sections were then lightly counterstained with hematoxylin, dehydrated, and coverslipped. Immunofluorescence (IF) analysis was performed using phosphorylation-specific rabbit polyclonal pAb anti-pTDP primary antibody (pS403/404 1:1000 Cosmobio), Alexa Fluor 488—conjugated secondary antibody (Life Technologies), and treatment for autofluorescence with Sudan Black 0.2% *w*/*v* in 70% ethanol before mounting with ProLong Antifade—DAPI mountant (Life Technologies) and coverslipping. Digital images were obtained using Life Technologies EVOS FL Auto imaging system (Life Technologies).

Statistical Analyses

To confirm discrimination of novel object by the animals during memory testing against a predefined chance level, one-sample Student's *t* test was used. For behavioral/molecular data comparison between two groups, unpaired Student's *t* test was used, with additional Welsch correction in cases where the variances were unequal between the two groups. For data involving comparison between more than two groups, one-way ANOVA was used. To check the effect of aging on miRNA upregulation upon learning, two-way ANOVA was employed. Significant ANOVA analyses were followed by post-hoc pair-wise comparisons. Tukey's or Sidak's post-hoc was used for pair-wise comparisons. Outliers were defined as values beyond two standard deviations from the group mean and were removed from the analyses. Significance was set at $p < 0.05$ for all tests and two-sided tests were performed. Statistical analysis was performed using GraphPad prism version 7 and verified by SPSS version 23. All graphs were drawn with GraphPad prism version 7.

Results

MiR-183/96/182 Is Decreased with Aging in the Mouse Hippocampus while Nuclear PP1 Activity Is Increased

To assess the role of miR-183/96/182 and PP1 in age-related cognitive decline, we trained young (4–5 months) and old (20–21 months) mice on a novel object recognition (NOR) task (Supplementary Fig. 1). We chose NOR because it depends on the hippocampus, is an efficient and reliable paradigm to induce long-term memory, and can reveal cognitive deficits associated with aging [34, 42]. Aged mice had impaired memory for objects compared to young mice when tested 24 and 48 h after training (Fig. 1a). In young mice, memory formation was associated with an increase in miR-183 and 182 and their precursor (pre-mir-183 and pre-miR-182) and primary (pri-mir-183-96-182) RNAs in the hippocampus 30 min after NOR training, but not in aged mice where only pri-mir-183-96-182 was increased (Fig. 1b, c). Since PP1 can regulate miR-183/96/182 biogenesis post-transcriptionally, we examined if the failure in miR-183/182 upregulation after NOR training is linked to changes in the level of PP1 [42]. Consistently, nuclear PP1 activity was significantly increased in the hippocampus of aged mice (Fig. 1d), while one of its inhibitors, the regulatory subunit PP1R1A, was decreased and its nuclear localization partner AKAP1 increased (Supplementary Fig. 2).

Increase in Nuclear PP1 Activity with Aging Impairs Microprocessor-Mediated Biogenesis of miR-183/96/182 Through R-SMADs

We next tested if the microprocessor processing of pri-mir-183/96/182 is affected by the increased nuclear PP1 or aging using renilla-firefly dual-reporter assays for pri-mir-183 and pri-mir-182 processing in mouse neuroblastoma (N2a) cells [2, 42]. Induction of senescence by hydroxyurea treatment (Supplementary Fig. 3) and PP1 overexpression decreased the microprocessor processing of pri-mir-183 (Supplementary Fig. 4) [10]. Overexpression of PP1 did not alter the expression of Drosha/DGCR8, two major components of the microprocessor complex (Supplementary Fig. 5), suggesting that they are not directly involved in the effect of PP1.

Since pri-mir-183-96-182 has a SMAD-binding element (SBE) and R-SMADs depend on phosphorylation at serine 463/465 for nuclear localization and interaction with the microprocessor component p68 [6, 7], we examined if R-SMADs are altered. Overexpression of a nuclear inhibitor of PP1 (NIPP1) increased R-SMAD 1/5 Ser463/465 phosphorylation in N2a cells (Supplementary Fig. 6), suggesting that nuclear PP1 can regulate the microprocessor processing of miR-183/96/182 by dephosphorylating SMADs. Consistently, pharmacological inhibition of transforming growth factor beta (TGF-β)known to lead to R-SMADs phosphorylation, decreased pre-mir-183 (Supplementary Fig. 7). Further, microprocessor processing of pri-mir-183 decreased when SMAD5 was knocked-down (Supplementary Fig. 8).

Overexpression of miR-183/96/182 in Hippocampus Reverses Age-Related Cognitive Deficits in Aged Mice

To assess the causal relationship between miR-183/96/182 and memory decline, we overexpressed miR-183/96/182 in

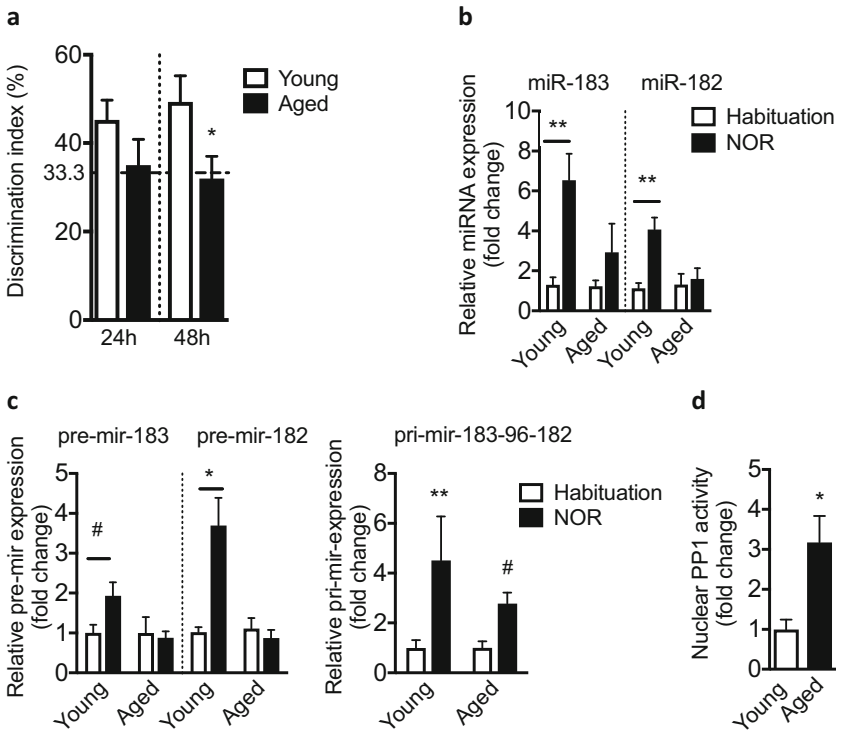

Fig. 1 Effects of aging on object memory and miR-183/182 expression. **a** Performance of young and aged mice was tested 24 and 48 h after novel object recognition (NOR) training and expressed as discrimination index. At both 24 and 48 h, only young animals demonstrate significant discrimination of the novel object (chance level set at 33.3%) (24 h—one-sample *t* test, control 33.3%, $p < 0.05$; 48 h—one-sample *t* test, control 33.3%, $p < 0.05$). Additionally, young mice discriminate novel object better than aged mice at 48 h (unpaired *t* test $*p < 0.05$). Young, $n = 9$ and aged, $n = 8$. **b** Young and aged mice were trained on an NOR task and hippocampus was collected 30 min after the end of training. NOR training increases miR-183 (left) and 182 (right) in young, but not aged mice (two-way ANOVA: miR-183: F 1, 17 = 3.399, $p = 0.0021$, post-hoc: young $**p < 0.01$, aged $p = 0.618$, miR-182: F 1, 17 = 11.04, $p = 0.004$, post-hoc: young $**p < 0.01$, aged $p = 0.978$). Young, $n = 6$ and aged, $n = 6$. **c** NOR training increases pre-mir-182 (left panel) in young but not aged mice, but increases pri-mir-183/96/182 in both young and aged mice (right panel) (two-way ANOVA: pre-mir-183: F 1, 15 = 1.555, $p = 0.23$, post-hoc: young $p = 0.15$, aged $p = 0.99$; pre-mir-182: F 1, 15 = 5.908, $p = 0.028$, post-hoc: young $*p < 0.05$, aged $p = 0.99$; pri-mir-183/96/182: F 1, 15 = 22.42, $p = 0.0006$, post-hoc: young $**p < 0.01$, aged $^{\#}p < 0.1$). Young, $n = 6$ and aged, $n = 6$. **d** Nuclear PP1 activity is increased in hippocampus from aged mice compared to young mice (unpaired *t* test: $*p < 0.05$). Aged, $n = 4$ and young, $n = 4$

area CA1 of the hippocampus in aged mice using a self-complementary adeno-associated virus (scAAV) vector expressing pre-miR-183/96/182 fused to GFP. Injected aged mice had memory performance comparable to young mice when tested 24 and 48 h after NOR training (Fig. 2a), suggesting that miR-183/96/182 can correct aged-related cognitive decline. Further, when exposed to environmental enrichment (EE), a paradigm known to reverse cognitive impairment in aged animals, nuclear PP1 activity was decreased while the expression of its inhibitors PP1R1A and NIPP1 was increased (Fig. 2b, c). EE also increased miR-183/182 and their precursors (Fig. 2d), confirming that this cluster can be dynamically regulated by memory-enhancing paradigms.

TDP-43 and FUS Regulate miR-183/96/182 Biogenesis

Emerging evidence suggests that changes in the expression and functions of miRNAs are associated with memory deficits in neurodegenerative disorders. This may result from altered expression of memory-relevant genes, in the case of early and subtle memory impairment, and possibly subsequent induction of genes favoring neuronal death [16, 43]. We examined if impaired biogenesis of miR-183/96/182 contributes to cognitive dysfunction associated with neurodegeneration using pri-mir-183/182 assays in vitro. Knock-down of TDP-43, a protein implicated in ALS and FTLD, impaired the microprocessor processing of pri-mir-183 and pri-mir-182 (Fig. 3a). This effect was in part rescued by pharmacological inhibition of PP1 (Fig. 3b). TDP-43 knock-down also increased nuclear PP1 activity (Fig. 3c) but did not change the expression of Drosha or DGCR8 (Supplementary Fig. 9), suggesting that TDP-43 is implicated in the regulation of miR-183/96/182 and PP1. Interestingly, knock-down of FUS, another RNA-binding protein implicated in ALS and FTLD, also impaired the microprocessor processing of pri-mir-183 and pri-mir-182 (Fig. 3d), suggesting that both TDP-43 and FUS can regulate miR-183/96/182 biogenesis. A

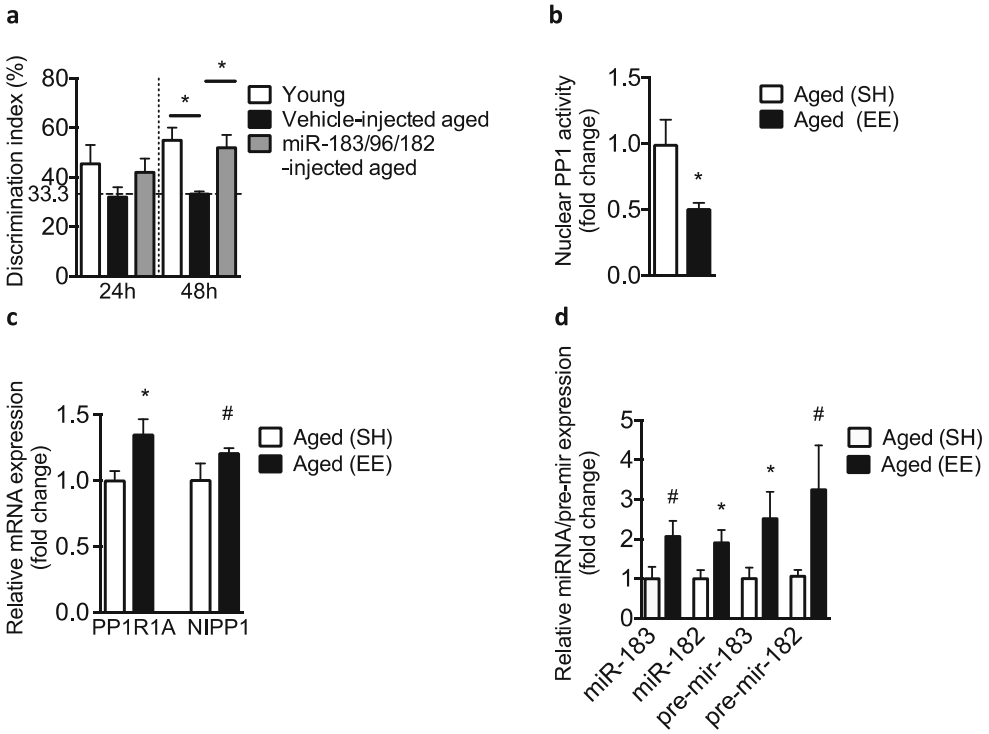

Fig. 2 Effects of miR-183/96/182 overexpression and EE on age-related memory deficits. **a** Overexpression of miR-183/182 in the hippocampus reverses memory decline in aged mice. Young mice have significant discrimination of the novel object at both 24 and 48 h (24 h: one-sample *t* test, control 33.3%, $p < 0.1$; 48 h: one-sample *t* test, control 33.3%, $p < 0.05$). miRNA-injected aged mice have significant discrimination of the novel object at 48 h (one-sample *t* test, control 33.3%, $p < 0.01$). MiRNA-injected aged mice show increased discrimination for the novel object when compared to vehicle-injected aged mice at 48 h (one-way ANOVA, $F\ 2, 14 = 6.018$, $p = 0.01$, post-hoc: young $^{*}p < 0.05$, miR-183/96/182 injected aged $^{*}p < 0.05$). Young, $n = 4$, vehicle-injected aged, $n = 8$, and miR-183/96/182 injected aged, $n = 8$. **b** Nuclear PP1 activity is significantly decreased in the hippocampus of aged mice exposed to EE (aged EE) compared to standard-housed aged mice (Aged SH) (unpaired *t* test, $^{*}p < 0.05$). Aged SH, $n = 8$ and aged EE, $n = 8$. **c** Increased mRNA expression of PP1R1A and NIPP1 in hippocampus from aged mice exposed to 4 weeks of EE compared to standard-house aged mice (unpaired *t* test: PP1R1A: $^{*}p < 0.05$, NIPP1: $^{\#}p < 0.1$). Aged SH, $n = 8$ and aged EE, $n = 8$. **d** Increased expression of miR-183 and miR-182, and their precursors in hippocampus from aged EE mice compared to aged mice in standard housing (unpaired *t* test: miR-183: $^{\#}p < 0.1$; miR-182: $^{*}p < 0.05$; pre-mir-183: $^{\#}p < 0.1$; pre-mir-182: $^{*}p < 0.05$). Aged SH, $n = 6$ and aged EE, $n = 6$

reduction in this miRNA cluster could therefore be a common mechanism underlying memory impairment in TDP-43 and FUS pathologies. To further confirm that FUS regulates miR-183/96/182, we checked the expression of miR-183/96/182 in FUS knock-in mice. MiR-183 and miR-96 expression was lower but miR-182 was normal in the cortex of FUS knock-in mice (Supplementary Fig. 10). Importantly, knock-down of alpha-synuclein (α-synuclein), another neurodegeneration-related protein, did not impair the microprocessor processing of pri-mir-183 or pri-mir-182, pointing to the specificity of miR-183/96/182 regulation by TDP-43 and FUS (Supplementary Fig. 11).

MiR-183/96/182 and PP1 Are Implicated in Human ALS and FTLD

We then explored the relevance of these findings for humans and focused on patients with ALS and FTLD, neurodegenerative conditions characterized by TDP-43 pathological aggregation (Fig. 4a) and cognitive dysfunctions [31, 36]. Consistent with our observation in aged mice, miR-183/96/182 expression was decreased in frontal cortex of patients with ALS while PP1γ, a predominantly nuclear isoform of PP1 was increased (Fig. 4b, c). MiR-183/96/182 expression was similarly decreased in the cortex of patients with FTLD with a comparable increase in PP1γ (Fig. 5a, b). Further, expression of several target genes of the cluster, known to be implicated in synaptic functions and cognitive processes, and previously shown to be affected by miR-183/96/182 expression [42], was increased in both ALS (Fig. 4d) and FTLD (Fig. 5c) patients, suggesting that reduced miR-183/96/182 expression in these disorders likely has functional consequences. Similar to data in vitro, R-SMADs appear as intermediates of the interaction between increased PP1 and decreased miR-183/96/182 in human subjects with TDP-43 pathology, since phospho-SMAD 1/5 showed a trend ($p = 0.06$) towards decrease in cortex from ALS and FTLD patients with TDP-43 pathology compared to age-matched controls (Fig. 5d).

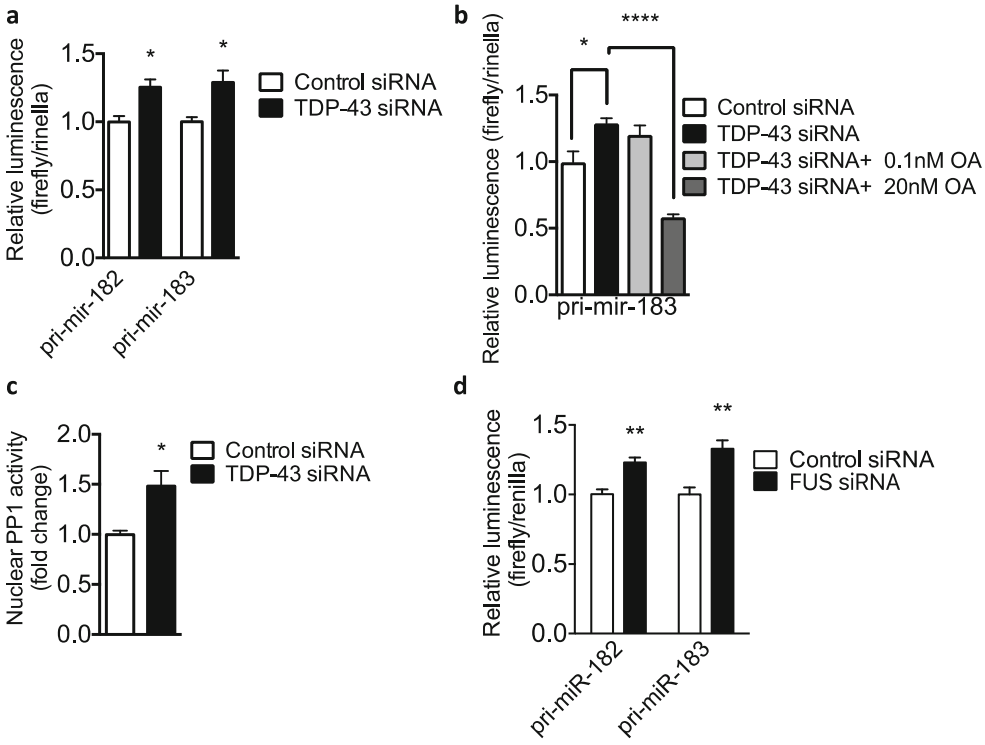

Fig. 3 TDP-43 and FUD regulate miR-183/182 biogenesis. **a** TDP-43 knock-down decreases microprocessor processing of pri-mir-183 and pri-mir-182 expressed as increased firefly/renilla luminescence (unpaired *t* test: pri-mir-183: $*p < 0.05$; pri-mir-182: $*p < 0.05$) in mouse N2a cells. Negative control siRNA, $n = 4$ and TDP-43 siRNA, $n = 4$. **b** Decreased microprocessor processing of pri-mir-183 on TDP-43 knock-down is reversed by 20 nM (inhibits PP1/PP2A) but not 0.1 nM okadaic acid (inhibits PP2A) (one-way ANOVA: F 3, 10 = 27.55, $p < 0.0001$, post-hoc: siRNA TDP-43 $*p < 0.05$, siRNA TDP-43+ 20 nM Okadaic acid $****p < 0.0001$). Negative control siRNA, $n = 4$; TDP-43 siRNA, $n = 3$; TDP-43 siRNA + 0.1 nM okadaic acid, $n = 3$; and TDP-43 siRNA + 20 nM okadaic acid, $n = 4$. **c** Nuclear PP1 activity is increased in mouse N2a cells treated with TDP-43 siRNA compared to control siRNA (unpaired *t* test, $*p < 0.05$). Negative control siRNA, $n = 4$ and TDP-43 siRNA, $n = 4$. **d** FUS knock-down decreases microprocessor processing of pri-mir-183 and pri-mir-182 expressed as increased firefly/renilla luminescence (unpaired *t* test: pri-mir-183: $**p < 0.01$; pri-mir-182: $**p < 0.01$) in mouse N2a cells. Negative control siRNA, $n = 4$ and TDP-43 siRNA, $n = 4$

Discussion

This study identifies impaired microprocessor processing of miR-183/96/182 as an important pathogenic mechanism of age-related memory decline and cognitive dysfunction in neurodegenerative disorders with TDP-43 and FUS pathological aggregation. While confirming a previously speculated role for PP1 and miRNAs in age-related memory disorders, the data newly demonstrate a mechanistic link between PP1, miR-183/96/182, TDP-43, and FUS.

The activity of nuclear PP1 in the brain may increase with aging due to an increase in the nuclear isoform of PP1, PP1γ, or to alterations in the mechanisms of PP1 regulation, e.g., by sub-cellular targeting, or control of substrate specificity or phosphatase domains activity [30]. We observed that the PP1 inhibitor PP1R1A is decreased whereas the PP1 nuclear scaffolding partner AKAP1 is increased in the hippocampus of aged mice, possibly explaining the increased activity of nuclear PP1 with aging. Further, TDP-43 knock-down also increased nuclear PP1, consistent with a previously demonstrated regulation of a closely related phosphatase PP2A by TDP-43 [33]. However, the increase in nuclear PP1 activity due to TDP-43 knock-down seems to involve a mechanism different from that of aging. Analysis of samples from cortex of ALS and FTLD patients with TDP-43 pathology revealed an increase in the mRNA of PP1γ, a predominantly nuclear PP1 isoform. This increase could reflect changes in the nuclear spliceosomal machinery, which includes TDP-43 as an integral component [8].

The results also show that aging and a decrease in TDP-43 and FUS impair the microprocessor-mediated biogenesis of miR-183/96/182. Although the biogenesis of a number of neuronal miRNAs is regulated by neuronal activity [43], these results suggest that aging can impair activity-dependent regulation of miRNAs through an increase in PP1. Similarly, they newly add miR-183/96/182 to the list of miRNAs regulated by TDP-43 and FUS. Previous reports have shown that TDP-43 can regulate microprocessor activity by stabilizing Drosha or through binding to certain pri-mirs (pri-mir-132, pri-mir-143, pri-mir-558, pri-mir-574) leading to their differential regulation by the microprocessor [9, 20]. Interestingly, two of these miRNAs; miR-132 and miR-574 are also implicated in

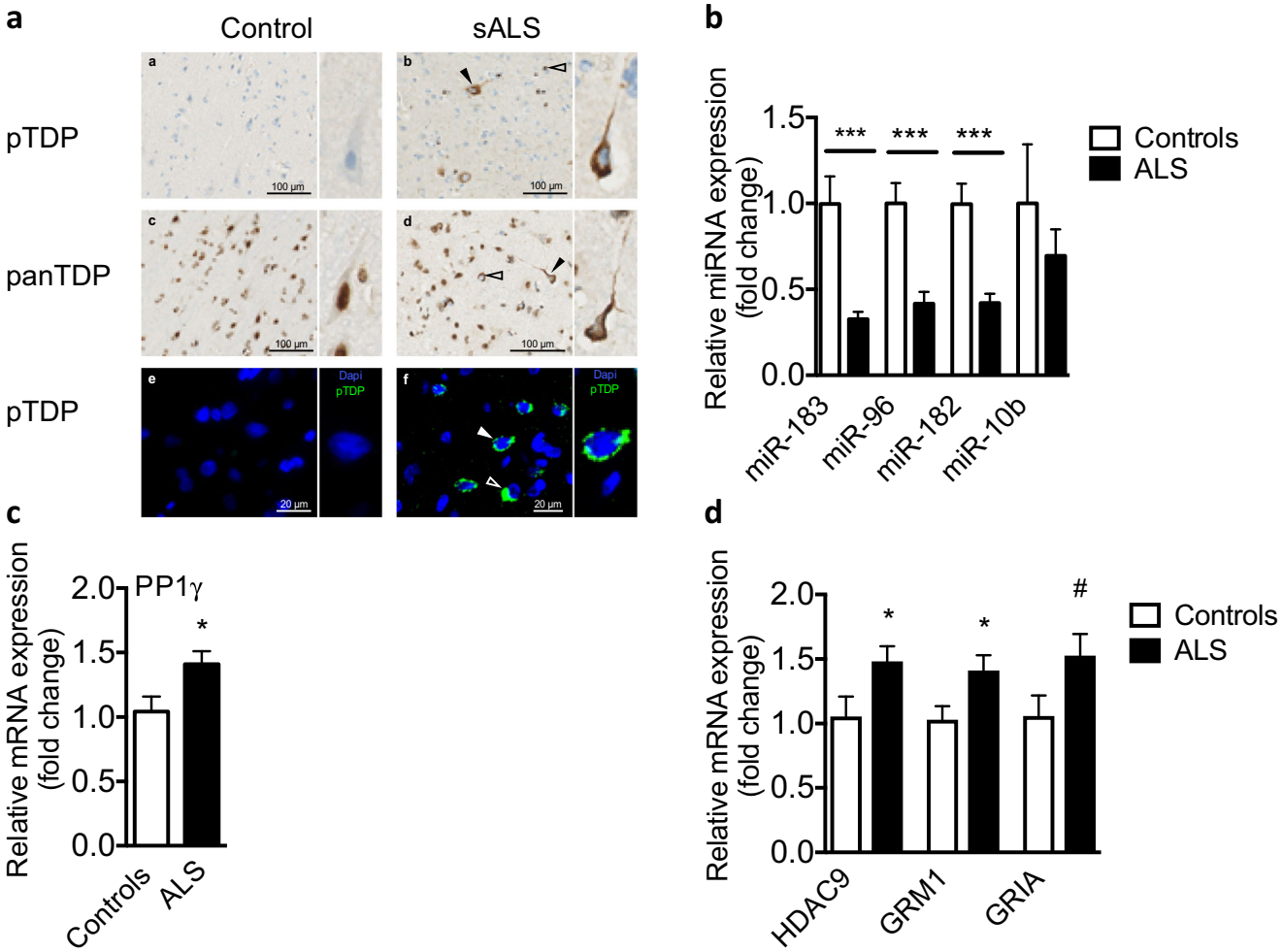

Fig. 4 miR-183/96/182 is altered in the brain of ALS patients with TDP-43 pathology. **a** Immunohistochemistry and immunofluorescence of phosphorylated TDP-43 and pan TDP-43 in patients with sporadic ALS and age-matched control subjects. **a**–**d** Sections of the motor cortex of controls (**a**, **c**) and patients with sporadic ALS (**b**, **d**) immunolabeled with antibody against phosphorylated TDP-43 (**a**, **b**), and pan TDP-43 (**c**, **d**). **e**–**f** Sections of the same regions labeled with phosphorylated TDP-43-specific antibody in immunofluorescence (pS403/404). Phosphorylated TDP-43 was not detected in control (**a**, **e**), and physiological TDP-43 shows clear nuclear immunoreactivity (**c**, adjacent magnification). In addition to normal TDP-43 staining, pTDP-positive cytoplasmic inclusions are detected in patients with sporadic ALS (**b**, **f**). Filled arrowheads indicate large skein-like inclusions in the cytoplasm and processes of pyramidal neurons (adjacent magnification). Empty arrowheads show small dense inclusions adjacent to nuclei (**b**, **d**, **f**). Note the absence of nuclear TDP-43 staining in cells with inclusions (**d**, adjacent magnification). **b** miR-183, miR-96, and miR-182 expression is decreased but not of a control miRNA miR-10b, in cortical tissue from patients with ALS compared to age-matched controls (unpaired *t* test: miR-183: $***p < 0.001$; miR-96: $***p < 0.001$; miR-182: $***p < 0.001$; miR-10b $p = 0.389$). Controls, $n = 8$ and ALS, $n = 10$. **c** PP1γ expression is increased in cortical tissue from patients with ALS compared to age-matched controls (unpaired *t* test, $*p < 0.05$). Controls, $n = 8$ and ALS, $n = 10$. **d** Expression of targets of miR-183/96/182 is increased in cortical tissue from patients with ALS compared to age-matched controls (unpaired *t* test: HDAC9: $*p < 0.05$; GRM: $*p < 0.05$; GRIA: $^{\#}p < 0.1$). Controls, $n = 8$ and ALS, $n = 10$

the regulation of cognitive processes in the brain [43]. Similarly, FUS has been previously shown to regulate the biogenesis of a sub-set of miRNAs, notably miR-9, miR-125, and miR-132 by modulating Drosha recruitment [29]. The simultaneous regulation of miR-183/96/182 and another miRNA critical for memory formation, miR-132, by TDP-43 and FUS suggests a functional homology between the two proteins for controlling memory formation by miRNAs.

The results further reveal R-SMADs as potential intermediates in the effect of PP1 on the microprocessor. SMAD proteins are downstream signaling molecules of TGF-β and bone morphogenic protein (BMP), which translocate to the nucleus upon activity-induced phosphorylation. Regulation of the microprocessor by R-SMADs involves a phosphorylation-dependent interaction with the microprocessor accessory protein p68 [1, 6, 7]. Control of nuclear SMAD1/5 phosphorylation by PP1, combined with the known role of R-SMADs in selectively regulating the microprocessor processing, makes them a likely mediator of the selective regulation of miR-183/96/182 by nuclear PP1. This

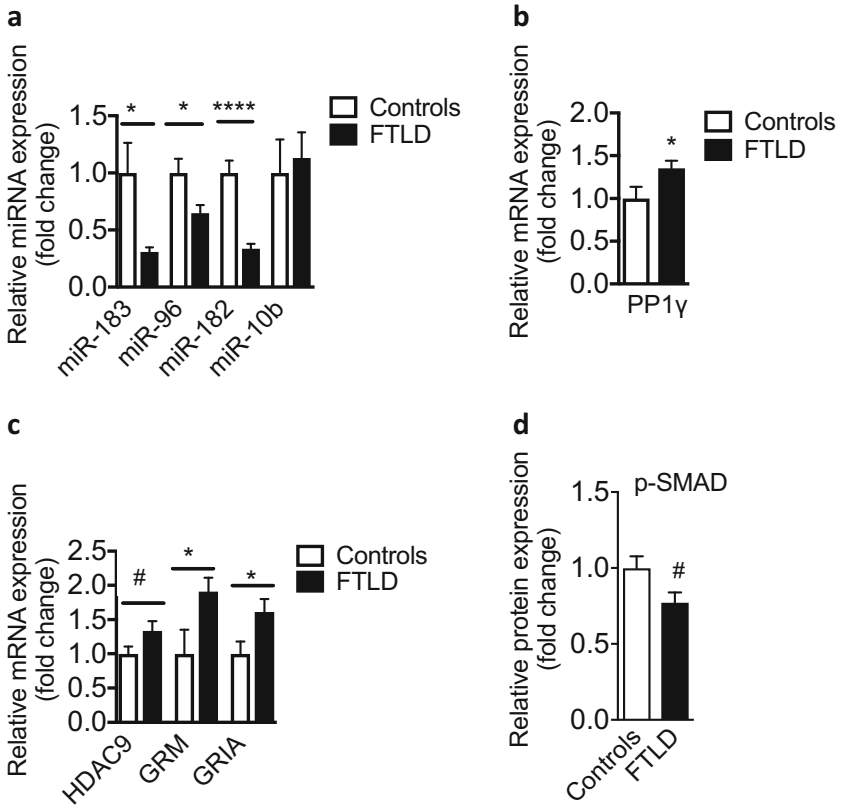

Fig. 5 miR-183/96/182 is altered in the brain of FTLD patients with TDP-43 pathology. **a** miR-183, miR-96, and miR-182 expression is decreased, but not of a control miRNA miR-10b, in cortical tissue collected from patients with FTLD compared to age-matched controls (unpaired *t* test: miR-183: $*p < 0.05$; miR-96: $*p < 0.05$; miR-182: $****p < 0.0001$; miR-10b $p = 0.737$). Controls, $n = 6$ and FTLD, $n = 12$. **b** PP1γ expression is increased in cortical tissue collected from patients with FTLD compared to age-matched controls (unpaired *t* test, $*p < 0.05$). Controls, $n = 6$ and FTLD, $n = 12$. **c** Expression of targets of miR-183/96/182 is increased compared to age-matched controls in cortical tissue collected from patients with FTLD (unpaired *t* test: HDAC9: $^{\#}p < 0.1$; GRM: $*p < 0.05$; GRIA: $*p < 0.1$). Controls, $n = 6$ and FTLD, $n = 12$. **d** p-SMAD expression is decreased compared to age-matched controls in cortical tissue collected from patients with FTLD (unpaired *t* test, $^{\#}p < 0.1$). Controls, $n = 5$ and FTLD, $n = 9$

possibility was investigated in several ways: (1) bioinformatic validation of the presence of SMAD binding element on pri-mir-183/96/182 (data not shown), (2) decreased pre-mir-183 upon inhibition of TGF-β, (3) selective effect of SMAD5 on microprocessor processing of pri-mir-183, (4) altered expression of miR-21, a SMAD-dependent miRNA, in N2a cells after PP1 manipulation (data not shown), and finally (5) decreased phospho-SMAD 1/5 in human ALS/FTLD samples with increased PP1γ and decreased miR-183/96/182.

Virus-mediated overexpression of pre-miR-183/96/182 in hippocampus reversed age-related cognitive decline in this study. Such correction establishes that the association between miR-183/96/182 and age-related cognitive impairment involves suppressed microprocessor-mediated biogenesis of these miRNAs by increased nuclear PP1, and that cognitive defects can be corrected if precursor miRNAs are replenished even when nuclear PP1 is increased. Similarly, EE, known to improve memory in aged rodents, increased post-transcriptional biogenesis of miR-183/96/182 and decreased nuclear PP1 activity in our study. A role for PP1 in age-related memory loss is also supported by the fact that aged rodents generally do not show memory deficits when trained on strong NOR protocols involving spaced learning [34], an effect shown to be associated with inhibition of nuclear PP1 [12]. These results highlight the potential of PP1- and miR-183/96/182-based modalities to delay dementia in the elderly. Indeed, multi-modal stimulation, an extrapolated equivalent of EE in humans, has shown promising results in reducing dementia symptoms in elderly with AD and minimal cognitive impairment [5, 26].

Finally, our results newly highlight a PP1-dependent mechanism for the regulation of miRNA biogenesis by TDP-43, a function that adds to its previously known action on Drosha and Dicer. This effect on miR-183/96/182 biogenesis could potentially be responsible for cognitive dysfunctions observed in TDP-43 pathologies. It may also be involved in motor dysfunctions observed in ALS since miR-183 and miR-182 have been suggested to play important roles in regulating muscular

gene expression in animal models of pathological muscle atrophy [17, 24].

Emerging evidence suggests that besides neuronal death, epigenetic mechanisms are responsible for early and persistent synaptic dysfunction and cognitive decline in the neurodegenerating brain. These epigenetic changes include increased expression and activity of HDACs [13]. Our results suggest that impaired biogenesis of memory-promoting miRNAs could be an additional non-genomic mechanism of cognitive impairment in neurodegenerative disorders. Future studies should consider a role for impaired biogenesis of additional miRNAs known to regulate memory formation, such as miR-132/212 [15], miR-335 [4], miR-12 [28], miR-124 [28], miR-181a [44], and miR-29b [21] in neurodegenerative disorders. Furthermore, cognitive alterations observed in ALS are pleiotropic with pronounced frontal-mediated behavioral dysfunctions in a vast majority of patients [41] and surprisingly low rates of depression despite physical disability [35]. Evidence suggesting a role for miR-183/96/182 in the regulation of other cognitive functions, such as stress response [27] and pro-depressive behavior [25] in mice suggests their potential contribution to broader cognitive phenotype of ALS, which warrants further investigations. In conclusion, we have identified a novel role for impaired microprocessor-mediated miRNA biogenesis that occurs downstream of several aging and neurodegeneration-related pathways in memory disorders, and may have important implications for prevention and/or treatment of dementia.

Acknowledgments We thank John Ravits (University of California, San Diego) for generously providing human autopsy tissues for analysis; Vinnie Kandra, Jennifer Brown, and Mattia Privitera for technical help; Jean-Charles Paterna and Jacek Krol for virus production, animal caretakers for mouse colony management.

Authors' contribution A. J. and I. M. M. conceived and designed the study, analyzed the results, and wrote the manuscript. A. J conducted behavioral experiments, molecular analyses of brain samples, and qPCR assays, and designed and executed in vitro experiments. B. T. W. performed stereotaxic injections and helped in the interpretation and drafting of in vivo data. E. A. K. conducted environmental enrichment and luciferase assays with A. J. F. L. performed Western blots and immunohistochemistry. N. G. assisted A. J. and E. A. K. in qPCR and luciferase assays. T. A. extracted RNA from human samples. M. P. provided human samples with TDP-43 pathology and critical input to the manuscript.

Funding Information This work was supported by the University of Zurich, the Swiss Federal Institute of Technology, the Swiss National Science Foundation (grant No. 31003A_153147/1). M.P. is supported by the Swiss National Science Foundation (grant No. PP00P3_144862 and NCCR RNA & Disease) and a Clinical Research Priority Program on Small RNAs by the University of Zurich.

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